Effects of biomembrane properties on cellular responses in an epicardial patch

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Abstract

Ischemic heart failure is the leading cause of death in the world. The disease is caused by coronary heart disease, in which the narrowed coronary arteries limit oxygen- and nutrient-rich blood from reaching the myocardial tissue. Obstructed arterial blood flow can cause myocardial necrosis and scarring. Scar tissue is non-contractile and poorly elastic. It can thus compromise the pumping capacity of the heart. Current medical and interventional therapies have only very limited efficacy to reduce myocardial scarring. Preclinical and clinical research efforts are underway to generate myocardial scar-reducing and regenerative therapies.

In the field of cardiac cellular therapies, the delivery of cells has conventionally been based on intramyocardial injections. However, epicardial patches have been demonstrated to reduce scarring and promote myocardial healing. In addition to merely being a carrier or cover for the cellular transplant, the biomembrane of the patch can also be considered as an active element for the patch's therapeutic activity. Thus, the properties of the biomembrane can have a major impact on both the cellular and the therapeutic tissue response.

The aim of this Master's thesis was to build a standardized test set up to study the properties of the biomembrane. Biomembrane permeability to small (glucose, lactate) molecules and different size proteins was investigated. In addition, the set up was modified to enable the investigation of biomembrane properties on the survival of the grafted cells. Finally, the test set up was evaluated by studying the properties of ProxiCor^{TM}, the biomembrane currently used together with autologous atrial micrografts (AAMs) in epicardial patch.

As a result, the set up was successfully constructed and characterized. The ProxiCor^{TM} membrane demonstrated permeability to both small molecules and proteins, and a stable pH was maintained across the membrane. ProxiCor^{TM} enabled traverse serum-induced proliferation of cells compared to the control impermeable membrane. Taken together, these results prove the functionality of the test set up and thus support its further development.

Keywords
atrial micrografts, biomembranes, cell therapy, epicardial biohybrid patch, heart failure, permeability

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Tiivistelmä

Iskeeminen sydämen vajaatoiminta on maailman johtava kuolinsyy. Sairauden aiheuttaa pitkäaikainen sydänlihaskudoksen hapen ja ravintoaineiden puute, jonka taustalla on usein sepelvaltimotauti. Tukkeuma sepelvaltimoissa estää hapen ja ravintoaineiden pääsyn sydänlihaskudokseen ja voi täten johtaa sydännekroosiin ja arpeutuneeseen sydänlihaskudokseen. Arpeutunut sydänkudos menettää joustavuutensa ja täten sydämen pumppausteho pienenee johtaen sydämen vajaatoimintaan. Vaikka iskeemistä vajaatoimintaa voidaan itsessään hoitaa monin eri tavoin ei arpeutuneelle kudokselle ole toistaiseksi olemassa tehokasta hoitomuotoa. Prekliinisiä ja kliinisiä tutkimuksia uusista hoitomuodoista on meneillään arpikudosta parantavan hoidon löytämiseksi

Solututkimuksissa sydänlihaskudoksen päälle asetettavien epikardiaalisten paikkojen on osoitettu lisäävän siirrettyjen solujen eloonjäämistä ja jäämistä sydänkudokseen sekä tehostavan parakriinisten vaikutusten tehoa verrattuna perinteisiin injektiohoitoihin. Soluja tukevan vaikutuksen lisäksi epikardiaalisissa paikoissa käytetty biomembraani on tärkeässä osassa paranemisprosessia ja sen ominaisuuksilla on suuri vaikutus hoitovasteeseen.

Tämän maisterintutkielman tarkoituksena oli rakentaa standardoitu koejärjestely, jonka avulla voidaan vertailla biomembraanien ominaisuuksia, kuten läpäisevyyttä pienille molekyyleille (glukoosi ja laktaatti), sekä erikokoisille proteiineille. Myös biomembraanin ominaisuuksien vaikutuksia siirretyistä soluista saataviin vasteisiin voitiin tutkia koejärjestelyä muokkaamalla. Koejärjestelyä testattiin tutkimalla ProxiCor™-biomembraania, jota käytetään eteiskorvakkeen soluista kehitetyssä epikardiaalisessa mikrosiirteessä.

Koejärjestely oli onnistunut ja sen avulla suoritettu ProxiCor™-membraanin koestus paljasti membraanin olevan läpäisevä sekä pienille molekyyleille että proteiineille. ProxiCor™-membraanin käyttö myös tasasi pH:ta tutkimusasetelmassa. Lisäksi ProxiCor™-membraanin käyttö mahdollisti seerumin indusoiman solujen jakautumisen läpäisemättömään kalvoon verrattuna. Saadut tulokset todistavat rakennetun koejärjestelyn toimivuuden ja tukevat täten sen jatkokehitystä.

Avainsanat
biomembraani, epikardiaalinen matriisi, eteiskorvake, läpäisevyys, soluhoito, sydämen vajaatoiminta

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Abbreviations

AAMs	autologous atrial micrografts
ACC/AHA	The American College of Cardiology and American Heart Association
ACE	angiotensin-converting enzyme
ARB	angiotensin-receptor blocker
BMMNC	bone marrow mononuclear cell
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CSC	cardiac stem cell
EDV	end-diastolic volume
EF	ejection fraction
EPC	endothelial progenitor cell
ESC	European Society of Cardiology
ESV	end-systolic volume
FBS	fetal bovine serum
HDL	high-density lipoprotein
HEK-293	human embryonic kidney 293 cells
HF	heart failure
HFmEF	heart failure with mid-range ejection fraction
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
HSC	hematopoietic stem cell
IC	intracoronary
IHD	ischemic heart disease
IM	intramyocardial
IV	intravenous

LDL	low-density lipoprotein
LVEF	left ventricular ejection fraction
MWCO	molecular weight cut-off
MI	myocardial infarction
MSC	mesenchymal stem cell
MW	molecular weight
PLA	poly (lactic acid)
RAAS	renin-angiotensin-aldosterone system
SIS-ECM	small intestine submucosa extracellular matrix
SM	skeletal myoblast

1. Background

1.1. Ischemic heart disease

1.1.1. Atherosclerosis and ischemic heart disease

Cardiovascular diseases, including ischemic heart disease (IHD) and heart failure (HF), are the leading causes of death worldwide (WHO, 2018). Annually, they are estimated to cause over 3.9 million deaths in Europe and exert an €210 billion burden on the healthcare costs in the European Union (Timmins et al., 2018).

In IHD, the flow of blood in coronary arteries is restricted. This restriction, usually caused by atherosclerosis, inhibits the transport of oxygen and nutrients to the heart tissue, ultimately leading to cell death and tissue damage. Eventually, atherosclerosis develops in everyone over time, but the development rate is determined by individual risk factors such as genetic predisposition or abnormalities, sex, age, cholesterol levels, type 2 diabetes, hypertension, and smoking (Kovanen & Pentikäinen, 2016).

Atherosclerosis occurs under the endothelium in the inner layer (intima) of blood vessels, and the arterial branch points are especially favorable for disease development (Kovanen & Pentikäinen, 2016). The most important event in the development of atherosclerosis is the accumulation of low-density lipoproteins (LDLs) into the intima (Figure 1). This accumulation begins already in childhood and continues throughout life.

Small amount of LDL enters the intima where the lack of capillaries or lymphatic system, and the attachment of the positively charged surface-proteins of the circulating cholesterol transporter, ApoB-100, to the negatively charged extracellular proteoglycans promotes retention of the LDL particles (Pentikäinen et al., 2000; Weber & Noels, 2011). In the intima the LDL particles are oxidized or modified by proteases or lipase enzymes. Macrophages in the intima recognize and phagocytose such modified particles, which are then scattered in the cells' lysosomes. Most of the released cholesterol is transported to the cell membrane and out of the cell, bit some of it end up as cytoplasmic esterified accumulates. Over time more and more cholesterol esters and accumulations are formed, and the macrophages turn into foam cells. (Weber & Noels, 2011; Kovanen & Pentikäinen, 2016.)

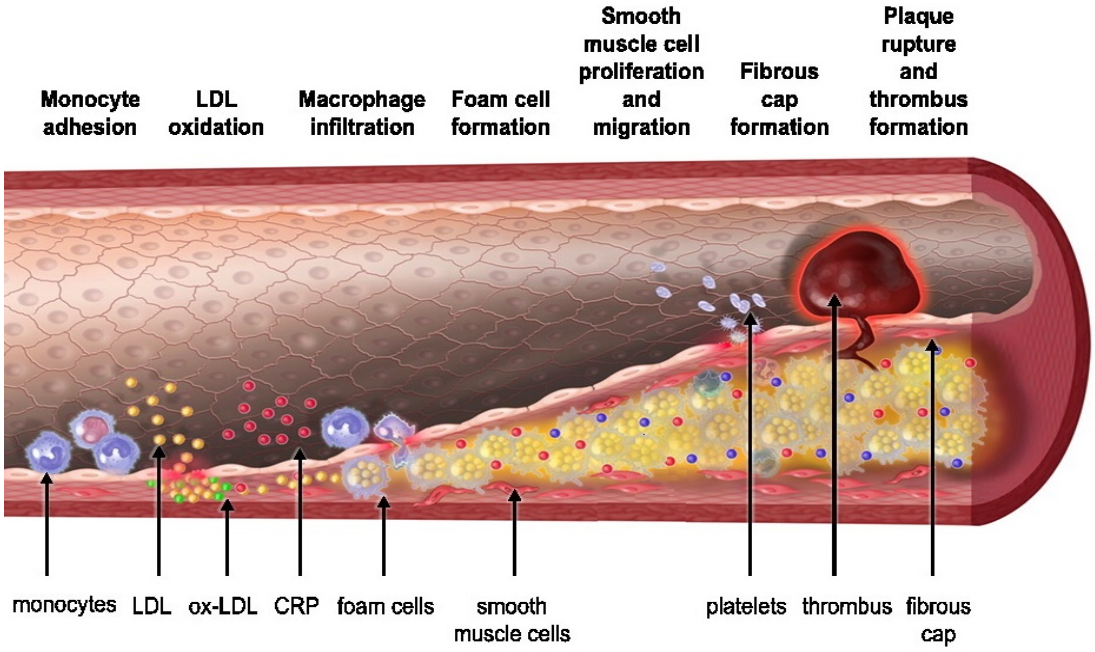

Figure 1. Atherosclerosis is a progressive disease which starts with accumulation of low-density lipoprotein (LDL) particles and macrophages into the intima of arteries. Over time, macrophages turn into foam cells, which form a fibroatheroma together with extracellular fat droplets. Myocardial infarction occurs when a plaque ruptures causing a thrombus and clogging the artery. Reproduced with permission from Nelson et al. (2017) under the Creative Commons Attribution 4.0 International License.

Some of the LDL particles are not phagocytosed by macrophages but end up in the musculoelastic layer of intima where these particles attach to elastin and form extracellular fat droplets. At the same time the foam cells die as a result of prolonged stress and cholesterol esters inside the cells are released. These free cholesterol esters together with fat droplets form a necrotic core. This intimal necrotic core keeps growing with the formation of new fat droplets and death of foam cells. (Kovanen & Pentikäinen, 2016.)

Atheroma forms when the necrotic core is formed at the site prone to atherosclerosis. When atheroma is isolated from the bloodstream by connective tissue, it is called fibroatheroma. These fibroatheromas, or atherosclerotic plaques, are characteristic of atherosclerosis. While the fibroatheroma expands, the connective tissue grows over it as a result of collagen synthesis and division of activated smooth muscle cells. (Weber & Noels, 2011; Kovanen & Pentikäinen, 2016.)

Coronary artery disease (CAD) is caused by hardening and narrowing of the walls of the arteries due the plaque formation. This leads to a stage at which the availability of oxygen and nutrients to the heart muscle is insufficient. The long-term lack of oxygen to the heart muscle tissue, due CAD or other causes, is called IHD and it may manifest as exertional chest pains (angina pectoris), or myocardial infarction (MI). Angina pectoris is chest pain that most often occurs during physical or mental exercise strain but is quickly relieved at rest. MI occurs if the blood circulation in a specific part of the heart muscle is totally blocked by thrombosis of atherosclerotic plaque. This can be caused by erosion or rupture of the plaque. (Xu, Bendeck & Gotlieb, 2016; Kovanen, Pentikäinen & Mustonen, 2016.)

The erosion-based thrombosis usually occurs with small fibroatheroma growing inward the artery. The rapid growth of plaque severely constricts the coronary artery, resulting in a situation where only a small blood clot can form a clog. This mechanism is behind chronic atherosclerosis with angina pectoris. A rupture ,in addition, is formed when a large fibroatheroma is growing outward of the artery, forming only thin connective tissue over the thrombogenic fibroadenoma tissue. The rupture reveals thrombogenic fibroadenoma tissue and activates the coagulation cascade, causing a large thrombus that clogs the artery. With ruptures, there are usually no symptoms in advance. (Kovanen, Pentikäinen & Mustonen, 2016.)

Prolonged ischemia can cause sudden cardiac death arrhythmia or necrosis of myocardial tissue. With necrosis, the dead cells are removed by macrophages and later on replaced by granulation tissue and eventually collagenous scar deposited by fibroblasts and endothelial cells. The scarred tissue reduces heart function by ventricle wall thinning and remodeling, which eventually leads to HF. (Xu, Bendeck & Gotlieb, 2016; Richardson et al., 2015.)

1.1.2. Heart failure and heart remodeling

HF is a condition where the pumping capacity of the heart is decreased and thus the blood supply to the body is inadequate. HF is often a result of decreased capacity of the ventricles to fill or eject blood either through reduced efficiency of myocardial contraction or structural abnormality.

After MI, the acute loss of myocardial cells results in an abnormal workload on the ventricle inducing dilatation which in turn renders the shape of the ventricle to a more spherical instead of elliptical form (Figure 2A). This remodeling does not only involve the infarcted area, but also the border zone and remote myocardium. Remodeling continues for months after the insult, and

eventually the changes in shape, orientation and length of the myocytes begins to affect the pumping capacity of the heart. (Bhatt, Ambrosy & Velazquez, 2017; Jessup & Brozena, 2003.) The spherical shape can also loosen the mitral leaflets and cause mitral regurgitation which further increases the workload of the left ventricle and, together with impaired conductivity and loss of elasticity from ischemia and fibrosis, contributes to the progression of disease (Abraham et al., 2002; Jessup & Brozena, 2003).

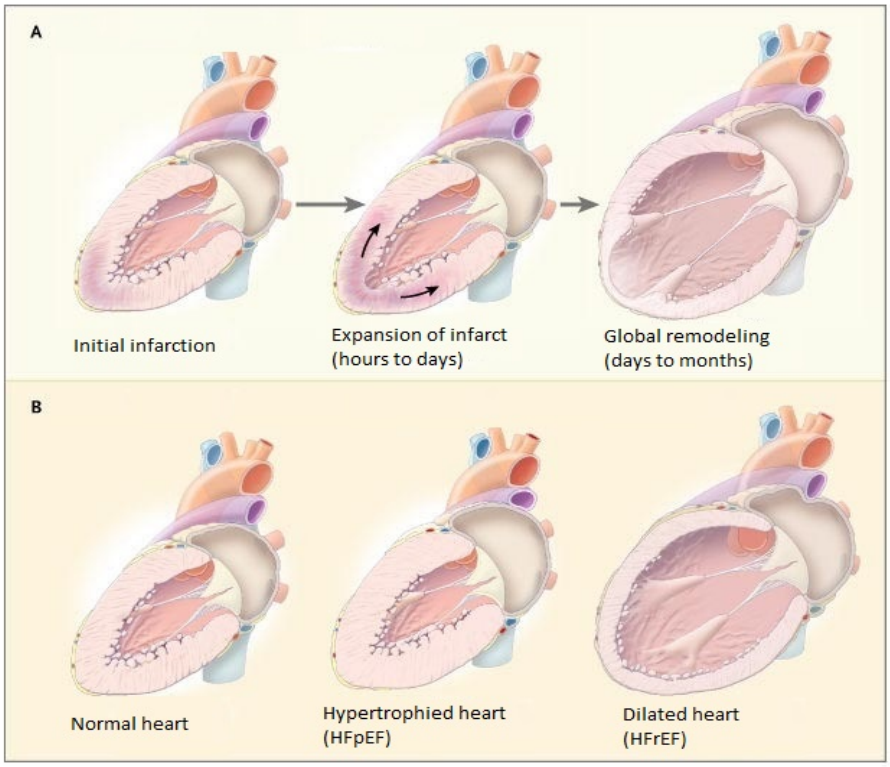

Figure 2. A. After myocardial infarction, the remodeling continues for months and changes the shape of the heart to more spherical. B. The heart failure with reduced ejection fraction (HFrEF) results in hypertrophied heart with normal left ventricular pumping capacity while the heart failure with preserved ejection fraction (HFpEF) has dilated heart and reduced contractility. Reproduced with permission from Jessup & Brozena (2003), Copyright Massachusetts Medical Society.

Ejection fraction (EF) measures the fractional efficacy of the heart to pump out blood at full contraction (end-systolic volume, ESV) after a full relaxation (end-diastolic volume, EDV). It is expressed as the percentage of the equation (EDV-ESV)/EDV. Heart failure can be divided into heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). The normal range of left ventricular ejection fraction (LVEF) is between 50% to 70%. An LVEF measurement under 40% is classified as HFrEF, while measurements of 41-49% are considered as borderline or mid-range HF (HFmEF). (Ponikowski et al. 2016.) HFpEF is

usually caused by thickening or stiffening of the left ventricle wall, resulting in a normal left ventricular contractility. Remodeling after MI more often results in a spherical shape of the heart with thin left ventricular walls and reduced LVEF (Figure 2B). (Jessup & Brozena, 2003.)

Myocardial dysfunction often activates the sympathetic nervous system and increases the neurohormonal activity (Bhatt, Ambrosy & Velazquez, 2017; Liem et al., 2014). This activation is a compensatory mechanism for reduced cardiac output and aims to raise the blood pressure. The most well-recognized mechanism is the renin-angiotensin-aldosterone system (RAAS). (Bhatt, Ambrosy & Velazquez, 2017; Jessup & Brozena, 2003.) Commonly, patients with HF have been treated with drugs such as beta-blockers, angiotensin-converting enzymes (ACEs), and angiotensin-receptor blockers (ARBs), which all either directly or indirectly affect the RAAS system (Bhatt, Ambrosy & Velazquez, 2017; Jessup & Brozena, 2003). However, Vergaro et al. (2019) demonstrated that neurohumoral activation occurs only in a subset of patients with HFpEF or HFmEF, and therefore neurohumoral antagonism is useful especially for patients with HFrEF but only for selected patients with HFpEF or HFmEF . In addition to RAAS, several vasoactive substances, such as norepinephrine and natriuretic peptide, are synthesized within the myocardium and thus those can alter the workload of the ventricle both in autocrine and paracrine manner (Bhatt, Ambrosy & Velazquez, 2017; Jessup & Brozena, 2003). Workload can also be altered with invasive assembly of compression assistants such as biomembranes or cardiac compression devices (Naveed et al., 2018).

1.2. Current treatment of heart failure

Treatment of HF depends on the severity of disease. The American College of Cardiology (ACC) and American Heart Association (AHA) (Yancy et al., 2017) and European Society of Cardiology (ESC) (Ponikowski et al., 2016) have published guidelines for the management of HF. In the ACC/AHA guidelines, the disease has been classified into four stages describing the progression of disease (Figure 3). In stage A, the patients have high risk factors without symptoms and in most severe stage D, the patients require immediate intervention. Thus, the disease is progressing, and by definition patients cannot go back to the earlier stage after entering the next one.

Especially in stages A and B, lifestyle changes the center of the treatment. Weight loss, physical activity, healthier food choices, and quitting smoking are the first steps to prevent disease onset. These changes aim to reduce the levels of cholesterol and sugar in circulating blood and lower the blood pressure. However, not all patients achieve benefits by modifying only lifestyle and, in some

cases, medication is required. Already in stage A, statin therapy should be prescribed in addition to dietary therapy to lower the serum cholesterol. ACE inhibitors and ARBs are used to prevent the production of angiotensin II or inhibit its receptor-mediated effects and thus to lower the blood pressure. (Yancy et al., 2017; Ponikowski et al., 2016.)

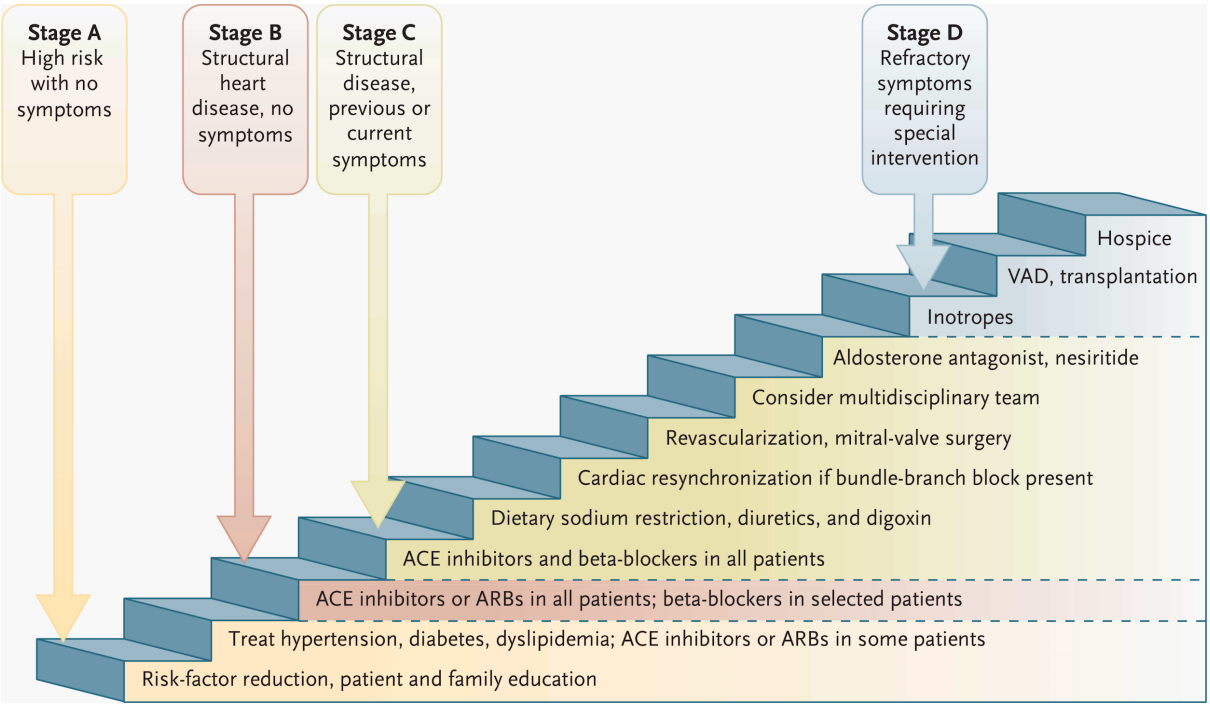

Figure 3. Heart failure is progressive disease and can be classified into four different stages. Traditionally the treatment is based on the severity of the disease. Reproduced with permission from Jessup & Brozena (2003), Copyright Massachusetts Medical Society

In stage B, there are structural changes like hypertrophy in the heart, but the patient remains without symptoms. This group also includes patients with previous MI, left ventricular systolic dysfunction or valvular heart disease. In stage B, ACE inhibitors and ARBs are routinely used for all patients. MI patients should also be prescribed with beta-blockers. Beta-blockers not only reduce heart rate by sympatholytic response but also decrease renin secretion in kidneys and thus lower the blood pressure. (Yancy et al., 2017; Ponikowski et al., 2016.) Beta-blockers can also enhance beneficial reverse remodeling of the myocardium (Khattar et al., 2001).

Patients with structural changes in the heart and with previous or current symptoms are classified into stage C. The treatment in stage C varies largely between individuals. ACE inhibitors and beta-blockers are used for all patients but usually there is a need for dietary sodium restriction or diuretics. Aldosterone antagonists are used to help to maintain the cardiovascular homeostasis if

symptoms remain after other therapies or if patients cannot use ACE treatment because of side effects.(Yancy et al., 2017; Ponikowski et al., 2016.) Digoxin has traditionally been used with atrial fibrillation patients to increase the contractility by affecting the Na^+/K^+-ATPase pump in the myocardial cells and to provide symptomatic benefit. However, use of digoxin has recently been linked with higher mortality and morbidity (Aguirre Dávila et al., 2019), and the benefits of digoxin may only be limited to selected patients. Serum digoxin concentration over 1.2 ng/ml has also shown to have a potential to contribute to ventricular tachyarrhythmia and bradyarrhythmia (Rathore et al., 2003).

In more advanced forms of stage C, invasive treatments come into play. Cardiac resynchronization treatment with a heart pacer is used with patients with present bundle-branch block. (Yancy et al., 2017; Ponikowski et al., 2016.) Pacers not only improve the left ventricular function but can also promote reverse remodeling (Gould et al., 2018). Percutaneous coronary intervention is used to open the blocked or narrowed arteries. In percutaneous coronary intervention, a small stent is usually implanted to a coronary artery to keep its lumen open. In coronary artery bypass by grafting (CABG), veins from lower limbs or arteries of the chest wall are used to circumvent blocked arteries and to supply blood to the myocardium.

The most severe stage is D, where special intervention is needed. Inotropes are used to help maintain myocardial contractility with ventricular assist devices. Heart transplantation can also be considered. At this point, hospice is the final option if other treatments have not been successful. (Yancy et al., 2017; Ponikowski et al., 2016.)

1.3. Cell therapies in the treatment of heart failure

Even though there are several treatment options for HF, we still have no effective treatments that targetedly modify myocardial scar tissue (Liang et al., 2019). In 1993, Koh et al. were the firsts to attempt to solve the problem with stem cells by creating intracardiac grafts from murine cardiomyocyte-like tumor cells. So far, several preclinical studies and clinical trials have been conducted based on different cardiac and non-cardiac cell sources (Figure 4) (Pfister et al., 2014).

In the early stages of disease, cell therapies may provide support to the arteries by modifying the inflammatory process. However, it is more likely that cell therapies redeem their place in the more advanced stages of disease in conjunction with invasive treatment. When considering autologous cellular therapies to elderly patients, one must take into account the age and the medical history of

the patient as these can have a great impact on the cells' therapeutic capacities. Also, we still suffer from lack of knowledge about the cellular factors that affect or modify the magnitude of the therapeutic response and thus contribute to the success of treatment.

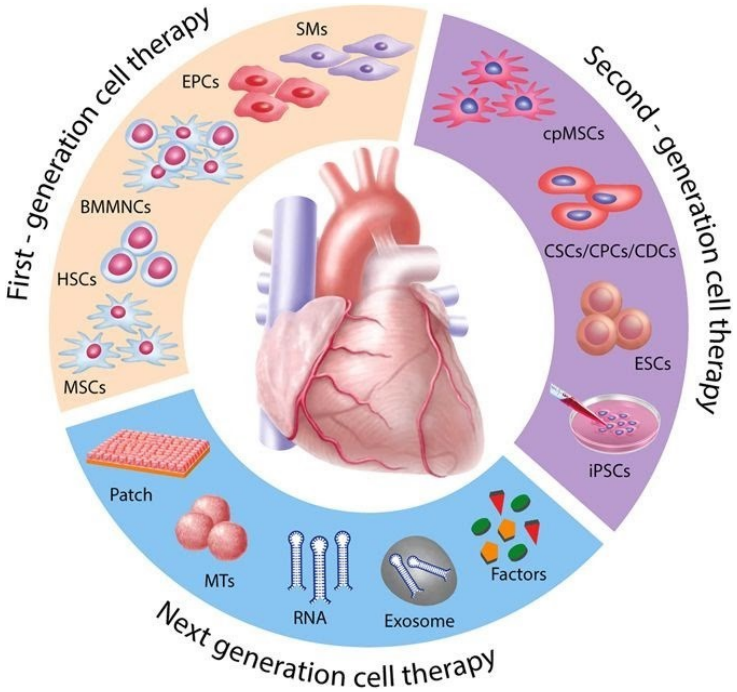

Figure 4. The results with non-cardiac stem cells, such as skeletal myoblasts (SMs) or bone marrow derived mononuclear cells (BMMNCs) have been mixed or only modest. Studies with cardiac stem cells (CSCs) have been promising but the efficacy and safety still needs to be further investigated. Currently the research trend has shifted towards cell free therapies such as patches, extracellular vesicles or microRNAs. Reproduced from Cambria et al. (2017) under the Creative Commons Attribution 4.0 International License.

Cell-mediated cardiac effects can be divided into two main categories: direct and paracrine effects. Direct effects consist of the niche-like environment provided by grafted cells and the transdifferentiation of these grafted cells into cardiomyocytes, endothelial cells and smooth muscle cells. These, together with recruitment and activation of resident stem cells, improve contractile performance. The recruitment and activation of resident stem cells is based on paracrine effects, which, in addition, play a role in reverse remodeling of myocardium by attenuating apoptosis, stabilizing scar formation by modulating matrix remodeling, and increasing capillary density by enhancing angiogenesis and vasculogenesis. (Pfister et al., 2014; Pagano et al., 2018.) In 2008, Korf-Klingebiel et al. reported secretome of bone marrow cells used in cardiac therapy, and in the same year Gnecchi et al. (2008) demonstrated that the beneficial effects of stem cell transplant depend more likely on paracrine factors secreted by grafted cells, rather than their differentiation.

The importance of paracrine factor has also sifted the research trend towards cell free therapies, which are based on small molecules such as microRNAs, extracellular vesicles, growth factors, and biomaterial patches (Cambria et al., 2017).

1.3.1. Non-cardiac stem cells

The first cells used in cardiac cell therapy were skeletal myoblasts (SMs) followed by bone marrow mononuclear cells (BMMCs), endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs).

SMs were the first cells entering the preclinical and clinical studies. Even though the SMs were shown to be resistant to ischemia and could be activated in response to muscle damage, in rodent models the SMs were not able to electrophysically couple to cardiomyocytes (Reinecke et al., 2000). In clinical studies, the SMs were shown to increase the risk of ventricular arrhythmias due to the defective coupling (Abraham et al., 2002). This led to the termination of the SM investigation.

Bone marrow-derived cells like BMMNCs, HSCs, and EPCs were widely studied cell sources due to their safety, high availability and easy isolation. The outcomes were mixed both in preclinical and clinical studies. Some clinical trials, such as TOPCARE-AMI or FINCELL, showed improvement in LVEF compared to control group while some trials, such as BONAMI and HEBE, reported no significant benefits (Hirsch et al., 2011; Leistner et al., 2011; Roncalli et al., 2011; Huikuri et al., 2008). In 2007, Zhang et al. suggested that the increase in angiogenesis due bone marrow cell treatment could be achieved through paracrine effects instead of myocardial differentiation.

MSCs are located in all postnatal organs, including the heart and can be isolated from bone marrow, adipose tissue, peripheral blood, and synovial tissue. Preclinical trials with MSCs were promising and trials such as POSEIDON and MSC-HF yielded positive outcomes in myocardial function (Hare et al., 2012; Mathiasen et al., 2019). MSC efficacy and safety still needs to be further investigated.

1.3.2. Cardiac stem cells

In 2002, Hierlihy et al. showed that the adult heart retains undifferentiated cardiac stem cells (CSCs) and nowadays research has shifted towards these. The sources for mesodermal lineage CSCs are the primary heart field the secondary heart field, and proepicardial organ. In addition to

these, there are ectodermal lineage-derived cells from cardiac neural crest. (Santini et al., 2016.) The best-known CSCs were identified by Beltrami et al. in 2003. They reported CSCs, which expresses c-Kit on their surface and proved to be clonogenic, self-renewing and multipotent. Later studies showed that these c-Kit+ -CSCs can be cardiomyogenic, originating from cardiac neural crest, or vasculogenic, originating from mesoderm (Hatzerigos et al., 2016). In the SCIPIO trial, the transplantation of c-Kit+-CPCs in patients with heart failure improved heart function, reduced infarct size, and increased viable tissue mass (Chugh et al., 2012). In 2017, Sharma et al. reported paracrine factors secreted by the c-Kit+ -CPCs after their transplantation in the infarcted myocardium and confirmed that the beneficial effects are due to paracrine pathways. In animal studies, c-Kit+ cells reduced infarct size and improved cardiac function through paracrine effects (Hodkinson et al., 2018). In addition, Sharma et al. noted that the paracrine factors secreted by rodent c-Kit+ -CPCs in comparison to human c-Kit+ -CPCs were different and thus the rodent models should not be used in studies for clinical applications. The cardiomyogenic availability of c-Kit+ -CPCs has later been questioned and those are claimed to be primarily endothelial progenitors (Elhelaly et al., 2019).

After observation of c-Kit+-CSCs, Laugwitz et al. (2005) reported Isl+ progenitor cells, which originate from both the secondary heart field and the cardiac neural crest, and can differentiate into several cell types, including cardiomyocytes and endothelial cells. In 2018, Foo et al. reported that injection of Isl+ cells into the hearts of NSG mice after MI preserved myocardial contractile function.

In the epicardium, there are pools of undifferentiated progenitor cells which, in murine models, are identified by expression of platelet-derived growth factor receptor-a (PDGFRa) and/or stem cell antigen-1 (Sca-1). These PDGFRa+/Sca-1+ -CSCs are reported to form colony-forming units and are able to differentiate into osteogenic, adipogenic, and chondrogenic derivatives. (Chong et al., 2011.) In 2019, Marunouchi et al. suggested that treatment with Sca-1+ cells preserved the cardiac pumping function and mitochondrial respiration better than c-Kit+ cells.

1.3.3. Infusion techniques in the delivery of cells

The cell delivery route is an important factor in the success of treatment. The most popular delivery routes are intramyocardial (IM), intracoronary (IC), and intravenous (IV) approaches (Figure 5).

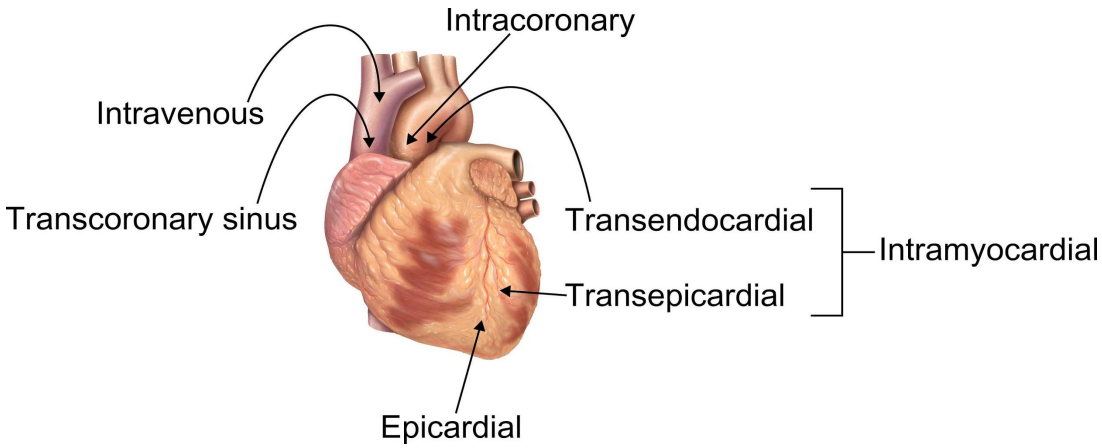

Figure 5. In addition to infusion-based techniques, there are epicardial patches, which enhance the cell retention and survival in the implantation area (Davies et al., 2016). Creative credits Lynch P.J. under the Creative Commons Attribution 2.5 Generic License.

The IM injections are targeted to the myocardium. Injection is usually directed to the border zone of the infarcted area, which has high impact on post-MI remodeling and where the blood supply ensures better survival of the injected cells (Kung et al., 2018; Olivetti et al., 1986). IM injections can be guided through the epicardium, endocardium or coronary vessels. While the epicardial IM injection is invasive and usually given during open heart surgery, endocardial IM injections can be performed with catheters through peripheral vessel access. Catheters are passed through peripheral vessels into the left ventricular cavity, where electromechanical methods are used to determine the border zone. While the endocardial procedure is less invasive than epicardial, uncontrolled electromechanical coupling has the potential to induce ventricular arrhythmias (Losordo et al., 2007). For trans-coronary IM injections, the catheter is passed from a peripheral vessel into the right atrium and from there to coronary sinus. While some studies report that IM approaches are the most efficient engraftments (George et al., 2008), other studies show that the grafted cells from IM injections are likely to form localized clusters within the myocardium (Fukushima et al., 2008), and that this heterogeneity may cause problems in conductivity and electrical impulse propagation resulting in generation of ventricular arrhythmias (Fukushima et al., 2007). The problem occurs especially with SMs which are contractile but rarely form gap junctions with cardiomyocytes (Fukushima et al., 2008).

In IC injection, the cell suspension is injected into the coronary circulation. The IC injection can be implemented using two different approaches; antegrade or retrograde. In the antegrade approach,

the catheter is inserted to a coronary artery from the proximal aorta while the retrograde approach uses coronary veins instead of arteria and thus enables cell delivery areas with lower arterial supply. The blood flow in the arteria or vein is blocked with an inflated balloon and the cells are injected to the distal side. Balloon is then deflated and the coronary flow is restored. Compared to IM approaches, IC injections are less invasive and most of the cardiologists are familiar with percutaneous technique. The major problem with IC techniques is poor cell retention in the injection area so that the cells are rapidly lost into the systemic circulation (Goussetis et al., 2006; Doyle et al., 2007). With IC techniques, there is also the risk of coronary embolism, especially when injecting larger cells such as MSCs (Vuillet et al., 2004).

IV injections are less invasive and less expensive compared to IM and IC approaches, but the cell migration to and retention in the heart are limited (Aicher at el., 2003). While some MSC types appear to have higher cardiac homing rates than others (Kraitchman et al., 2005), new strategies must be developed to improve cardiac retention.

1.3.4. Epicardial patches

In addition to infusion techniques, cells can be delivered onto the heart's outer surface, epicardially. Epicardial delivery can be achieved using, for example, cell sheets or epicardial patches, in which the cells are delivered with a biomembrane. An epicardial patch is then transplanted onto the myocardial tissue. While the epicardial technique as such requires open heart surgery, it offers distinct advantages over other delivery routes. In rat models, the cell retention and survival are increased compared to IM injections (Tano et al., 2014) and the paracrine molecules reduced fibrosis and increased angiogenesis even though the grafted cells were not reported to precipitate into cardiomyogenesis (Hamdi et al., 2014). In the advanced stages of disease, transplantation of the patch can be combined with implantation of a ventricular assist device and/or direct cardiac compression devices and thus support the circulation by compression and mechanically restrain the heart (Naveed et al., 2018; Xie et al., 2020).

However, the scaffold, which is used in the patch, is not only just a passive structure supporting the patch and providing protection to the cells, but it can also be considered as an active contributor to the therapeutic effects resulting from the patch treatment. The biomaterial scaffold is not a permanent insert, and it will eventually be resorbed and degraded in the body. However, the degradation products may have a great impact on the patch microenvironment. The natural based scaffolds, such as traditional porcine small intestine submucosa extracellular matrix (SIS-ECM), are

biologically active, promoting cell adhesion and growth. The degradation products of SIS-ECM have been shown to be antimicrobial (Sarikaya et al., 2002), promote vascularization, and stimulate endothelial cell recruitment *in vivo* (Li et al., 2004). In addition to SIS-ECM, ECM bioscaffolds can be produced from other tissues sources, which are considered to have potential for cardiac repair and regeneration. Table 1 presents several natural based scaffolds, which are already commercially available.

Table 1. A partial list of commercially available ECM bioscaffolds. Reproduced with permission from Pattar, Hassanabad & Fedak (2019) under the Creative Commons Attribution 4.0 Lisence

Bioscaffold product	Company	Donor Source	Tissue source	Fixation Method
AltiPly®	Aziyo Biologics, Inc.	Human	Placental	Natural
CardioCel	Admedus Ltd.	Bovine	Pericardium	ADAPT®, glutaraldehyde-based
CorMatrix®	CorMatrix Cardiovascular, Inc.	Porcine	Small Intestinal submucosa (SIS)	Natural
Edwards Pericardial Patch	Edwards Lifesciences Corp.	Bovine	Pericardium	XenoLogiX™, glutaraldehyde-based
Matrix Patch™	AutoTissue Berlin GmbH	Equine	Pericardium	--
Miroderm	Miromatrix Medical, Inc.	Porcine	Liver	Natural
No-React Pericardial Patch	BioIntegral Surgical, Inc.	Bovine	Pericardium	No-React®, glutaraldehyde-based
NuShield	Organogenesis, Inc.	Human	Placental	--
Peri-Guard®	Synovis Life Technologies, Inc.	Bovine	Pericardium	Glutaraldehyde-based
PhotoFix™	Cryolife, Inc.	Bovine	Pericardium	Dye-mediated photo-oxidation
SJM™ Pericardial Patch	St. Jude Medical, Inc.	Bovine	Pericardium	EnCap™, glutaraldehyde-based
Tyke®	Aziyo Biologics, Inc.	Porcine	Small Intestinal submucosa (SIS)	Natural
Vascutek Pericardial Patch	Vascutel Ltd.	Porcine	Pericardium	Glutaraldehyde-based
XenoSure®	LeMaitre® Vascular	Bovine	Pericardium	Glutaraldehyde-based
				-- , *information not available*

Bioscaffolds can also be fabricated from synthetic materials. The use of synthetic components may lead to improved surface properties and mechanical tolerance (Pattar, Hassanabad & Fedak, 2019). However, some of the synthetic materials, such as the commonly used polyglycolic acid (PGA) and polylactic acid (PLA), are degraded through hydrolysis and yield acidic degradation products (Avérous, 2008). These degradation products may lower the microenvironment's pH, cause necrosis of the target tissue cells and thereby inhibit or limit healing.

In addition to just supporting the cell delivery, the membrane can be designed to release desired factors such as angiogenesis-enhancing agents or drugs upon degradation. E.g. in 2015, Wei et al. evaluated the epicardial delivery of follistatin-like 1 (Fstl1) protein into the injured heart in three-dimensional collagen nanofibrillar patches with positive outcomes. However, the content of the patch can be more complex than just one specific agent. In 2017, Fleischer et al. reported a multifunctional cardiac patch which consists of various eletrospun albumin fiber layers with

different functions. The patch included substances such as cardiac cells, growth factors and anti-inflammatory drugs and shared mechanical properties similar to heart. In the future, this kind of multilayer scaffold may be used to protect not only the heart but also other tissues, including the lung and the liver (Fleischer et al., 2017).

1.3.5. Autologous atrial micrograft transplantation

Atrial appendages are extensions to the atriums. They slightly increase the atrial volumes, and act as decompression chambers during ventricular contraction (Al-Saady, Obel & Camm, 1999). The atrial appendages have been used as sources for stem cells already in 2012 SCIPIO trial (Chugh et al., 2012). In 2013 Koninckx et al. identified new CSCs from the atrial appendages. These cells had high aldehyde dehydrogenase (ALDH) activity and expressed CD34 on the cell surface but lacked CD45 and c-Kit expression. These $ALDH^{POS}CD34^{POS}CD45^{NEG}$ CSCs were named cardiac atrial appendage stem cells since they constituted a unique phenotype compared to the previously described CSCs.

Cardiac atrial appendage stem cells are reported to promote angiogenesis and be able to differentiate into functional cardiomyocytes (Koninckx et al., 2013; Fanton et al., 2016). The autologous atrial micrografts (AAMs) treatment is harnessing these features to treat scarred myocardium after MI. The AAMs patch procedure has been presented in studies by Nummi et al. (2017) and Lampinen et al. (2017) (Figure 6).

The AAMs patch is made in the operating room during on-pump CABG surgery. In the surgery, the central venous cannula of the heart-lung machine is inserted through the atrial appendage (El-Sherief et al., 2013). The patient's medical history and clinical evaluation determine whether the right or left atrial appendage is used. With atrial fibrillation patients, who cannot use anticoagulant therapy, the left atrial appendage can be closed and amputated. Thus, for these patients it is natural to isolate the micrografts from the left atrium appendage. For patients without atrial fibrillation, the right atrium appendage is used for micrografting. During cannula insertion, a piece of atrial appendage is removed for the generation of micrografts and subsequent utilization in the AAMs patch.

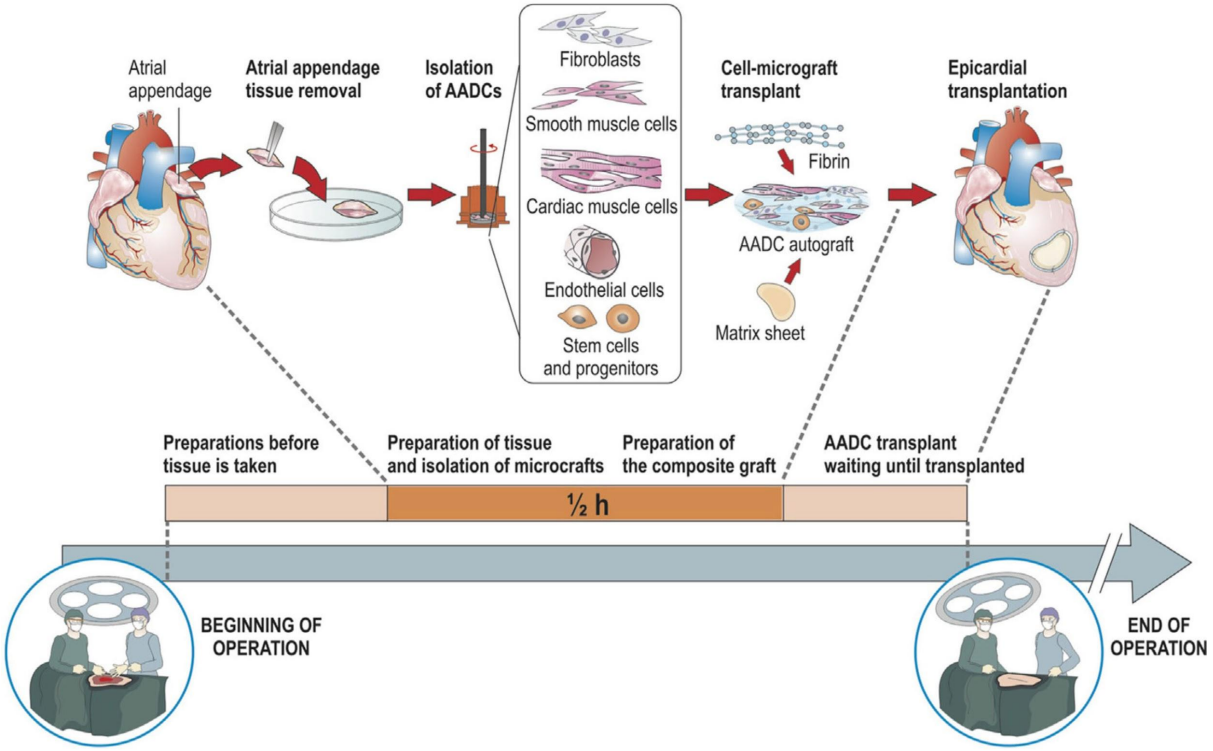

Figure 6. During coronary artery bypass by crafting (CABG) surgery, a small piece of atrial appendage is removed and homogenized into micrografts. These micrografts are then placed over the biomembrane and mounted on the myocardial scar tissue. Picture reproduced from Lampinen et al. (2017), with permission from the copyright holder Elsevier (License number 4785240546664). AADC= atrial appendage derived cells.

A tissue piece is mechanically homogenized into micrografts and these micrografts are placed over the biomembrane material to form a patch. The AAMs patch is mounted on the myocardial scar tissue identified preoperatively using cardiac magnetic resonance imaging (MRI). (Lampinen, 2017; Nummi, 2017.) Under the AAMs patch, the micrografts produce paracrine factors as previously discussed. These factors, such as growth factors, cytokines and extracellular vesicles, act on the underlying damaged myocardium to facilitate cardiac repair and angiogenesis, as well as to activate antifibrotic signaling pathways. The AAMs patch has been shown in experimental animal studies to heal scar tissue and restore tissue elasticity (Xie et al., 2020).

The currently used membrane in AAMs patch is the ProxiCor™ (Aziyo Biologics, Richmond, CA, USA), which is made of SIS-ECM. The ProxiCor™ is dense and considered to be impermeable. With this membrane it has been considered that the harmful metabolites produced by the cells of the graft, such as lactate, remain under the membrane, making the environment acidic and unfavorable to the cells. Meanwhile, outside the membrane the pericardium fluid contains a lot of energy-rich substances, such as glucose, which would be a natural source of energy for the

micrografts under the membrane (Figure 7). However, several studies (Gnecchi et al., 2008; Pfister et al., 2014; Pagano et al., 2018; Sharma et al., 2017) demonstrates that the positive effects of grafted cell are based on paracrine effects and thus the scaffold should retain the secreted proteins under the patch to achieve a positive effect on myocardial scar tissue.

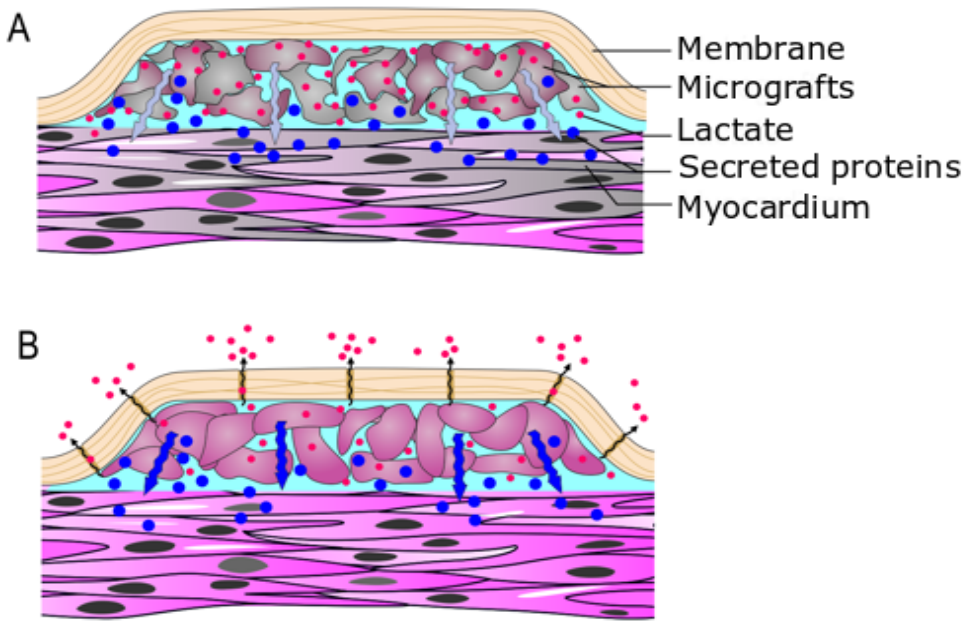

Figure 7. A. With impermeable biomembrane, harmful metabolites retain under the patch making the environment acidic. The unfavorable environment affects the grafted cells and restrains the therapeutic effect from secreted proteins. B. With permeable biomembrane, the harmful metabolites can sift out of the patch and thus boosts the cell viability and the healing properties of grafted cells.

2. Aims

Even if we know that biomembranes can have a tremendous impact on the cell responses in epicardial patches, the factors as well as information on how the biomembranes support healing, are still lacking attention.

The aim of this Master's thesis was to build a standardized test set up to study membrane properties. The test set up was evaluated by investigating the permeability of ProxiCor™ and to study how the biomembrane properties affect cell viability and proliferation when separated from the pericardial space and fluid in cardiac patches.

3. Materials and methods

3.1. Membrane permeability test set up

The membrane to be examined was placed into a 3.4 ml side-by-side diffusion chamber with 12 mm orifice diameter (PermeGear Inc., Hellertown, PA, USA). The left chamber was called a donor cell and the right chamber a receptor cell. The donor cell was considered to mimic the space between the patch and the epicardial surface and the receptor chamber was considered to mimic the space outside the patch (the pericardial extracellular fluid). Magnetic stirrers were inserted into both cells to maintain the movement of solutions. The test set up was kept at 37 °C with a water jacket heater. The diagram of the test set up is shown in Figure 8.B. In addition to ProxiCor™, Nunc™ polycarbonate membrane inserts (ThermoFischer Scientific, Waltham, MA, USA) with 0.4 µm pores were considered as positive control membrane and PARAFILM® (Bemis Company Inc., Neenah, WI, USA) as a negative control membrane.

To study small molecule movement, glucose and lactate were chosen as model molecules. Ringer's lactate solution (J67572, Alfa Aesar,Haverhill, MA, USA) was diluted with PBS (A099400100, Aniara Diagnostica, West Chester Township, OH, USA) to form a solution containing 2 mM lactate. D(+)-glucose (A16828, AlfaAesar) was dissolved with PBS to form a solution containing 5 mM D(+)-glucose.

To study protein retention, 25 µl of Precision Plus Protein™ Standard (1610374, Bio-Rad Laboratories, Hercule, CA, USA) and 25 µl of Spectra™ Multicolor Low Range Protein Ladder (26628, ThermoFischer Scientific) were added to the lactate solution. The protein mixture covered the molecular weights from 1.7 kDa to 250 kDa. The lactate-protein solution was added into the donor cell and the glucose solution into the receptor cell.

Measurements were conducted real-time for 24 hours. Glucose and lactate concentrations were measured with a LV5 biosensor (Jobst Technologies GmbH, Freiburg, Germany) and BioMON4 software (v.4.15.0, Jobst Technologies GmbH) from the receptor cell. pH stability in the donor cell was controlled with WTW inoLab® Multi 9310 IDS with SenTix® Micro 900 electrode (Xylem Analytics, Weilheim, Germany) through a MultiLab® Importer (v.1.22, Xylem Analytics). The protein movement was measured from the receptor cell as absorbance with Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at the 280 nm wavelength. The layout of meters is presented in Figure 8A.

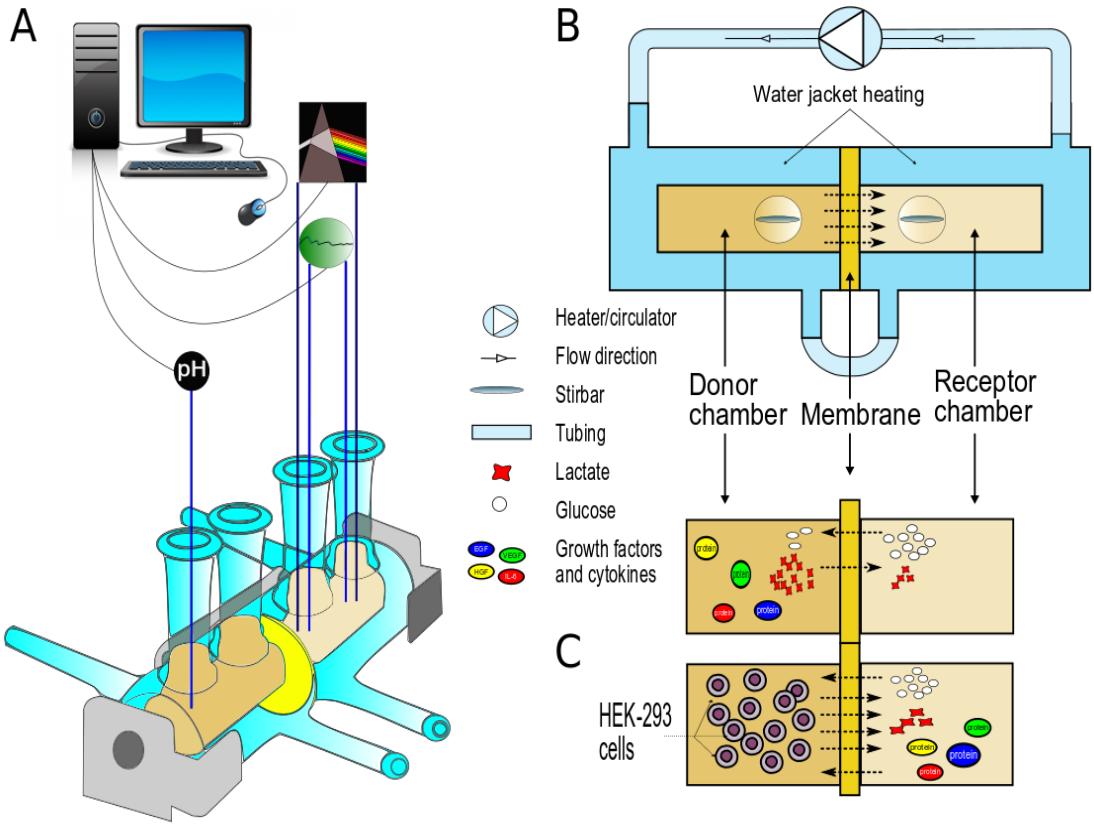

Figure 8. A. The decrease in glucose concentration and the increase in lactate concentration were measured from the receptor chamber with a biosensor. The protein movement into the receptor chamber was investigated as change in absorbance at 280 nm with a spectrophotometer. The stability of pH was monitored from the donor chamber. All measurements were conducted in real-time. B. The lactate and protein solution was inserted to the donor chamber, which was considered to mimic the space inside the patch. The glucose solution was inserted into the receptor chamber, which was considered to mimic the space outside the patch. The test set up was kept at 37 °C with a water jacket heater and the movement of solutions were maintained with magnetic stirrers. C. HEK-293 cell solution was placed into the donor chamber and cell viability and cell cycle analysis were conducted after 24 h incubation.

After the incubation, the solutions were collected from both chambers and concentrated with Microsep Advance Spin-ultracentrifugation units with 1 kDa molecular weight cut-off (MWCO) (MCP001C41, Pall Corporation, New York, NY, USA). Samples were then run through SDS-Page (80 V, 90 min.) to confirm by in-gel imaging using Coomassie Brilliant Blue R-250 (161-0436, Bio-Rad) the size of proteins on both sides of the membrane.

3.2. Cell viability and proliferation

Human embryonic kidney 293 cells (HEK-293, ATCC® CRL-1573™, LGC Standards GmbH, Wesel, Germany) were cultured in Leibovitz L-15 media (BE12-700F, Lonza Group AG, Basel, Switzerland) with 5 % fetal bovine serum (FBS) (Gibco™ 10500-065, ThermoFischer Scientific) , 1 % L-Glutamine (Gibco™ 25030081, ThermoFischer Scientific), 1 % Antibiotic-antimycotic (Gibco™ 15240096, ThermoFischer Scientific) and 5 mM glucose (Gibco™ A24940-01, ThermoFischer Scientific) additions. Cells were cultured at +37 °C in an ambient humidified air atmosphere without CO_2 supplementation.

To determine the optimal conditions for viability and proliferation testing, the HEK-293 cell cultures were kept in serum starvation for four different periods (2 days, 3 days and 7 days). After serum starvation, four FBS additions of varying strength (0 % addition, 1% addition, 5 % addition and 10 % addition) were added to the culture flasks for 24 hours. A normal cell culture without serum starvation and with 5% FBS addition was kept as a control. The viability was measured after serum incubation with Countess™ II automated cell counter (Applied Biosystems™, ThermoFischer Scientific) with trypan blue staining and cell cycle analysis was conducted by BD Accuri™ C6 flow cytometer (BD Biosciences, Frankin Lakes, NJ, USA) with propidium iodide (PI) staining. The conditions with strongest effects on cell proliferation were chosen for further testing.

A volume of 3.4 ml HEK-293 cell solution ($6x10^6$ cells/ml) was added to the donor chamber in Leibovitz L-15 media without serum addition. Leibovitz L-15 culture media with 10 % FBS addition was added into the receptor chamber (Figure 8C). The chambers were separated by a membrane under study. Cells and media were collected from donor and receptor chambers after a 24-hour incubation. The cell viability was analyzed before and after incubation and cell cycle analysis was conducted after incubation.

3.3. Data Analysis

The movement rate of small molecules, protein retention and pH stability were analyzed with GraphPad Prism 8 (v8.4.1, GraphPad Software, San Diego, CA, USA). After the identification of outliers, the glucose and lactate curves were smoothened against 15 neighbour measurements. The intensity of lines in the SDS-page were analyzed with ImageJ Fiji (Rev. 24.1.2020) (Schindelin, Arganda-Carreras & Frise et al., 2012). Cell viability information was analyzed with GraphPad

Prism 8 and the distribution of cell cycle phases was analyzed with BD FlowJo™ software (v10.6.2, BD Biosciences).

4. Results

4.1. Permeability of ProxiCor™

4.1.1. Small molecule movement

ProxiCor ™ permeability for small molecules was analyzed by measuring the changes in glucose and lactate concentrations from the receptor chamber. The stability of pH was also recorded. The permeability of ProxiCor™ was compared to the positive and the negative control membranes. As a result, the ProxiCor™ found to be permeable both for glucose and lactate. The pH remained stable regardless of the membrane .The results from glucose, lactate, and pH measurements are represented in Figure 9.

4.1.2. Protein retention

The protein movement from donor chamber to receptor chamber was inspected by measuring the changes in absorbance at 280 nm from receptor chamber every 5 minutes. The permeability of ProxiCor™ was compared to the positive and the negative control membranes. The results from absorbance measurements are represented in Figure 10. As the absorbance measurements with ProxiCor™ were higher than with positive control, the amount of proteins passed was not directly readable from the change in absorbance. Thus, the samples from donor and receptor chambers were run through SDS-page to investigate the proteins on both side of the membrane and further examine the size distribution of proteins passed through (Figure 11A). As a result, the ProxiCor™ found to be permeable also to proteins within studied range.

As the absorbance measurements with ProxiCor™ were higher than with positive control, there was an assumption that ProxiCor™ itself releases proteins. To confirm that assumption, a piece of ProxiCor™ was incubated in 3,4 ml 0.9% NaCl (BP358-1, ThermoFischer Scientific) for 24 hours. After incubation the liquid was collected and concentrated and ran through SDS-page (Figure 11B). Results confirmed the assumption of released proteins.

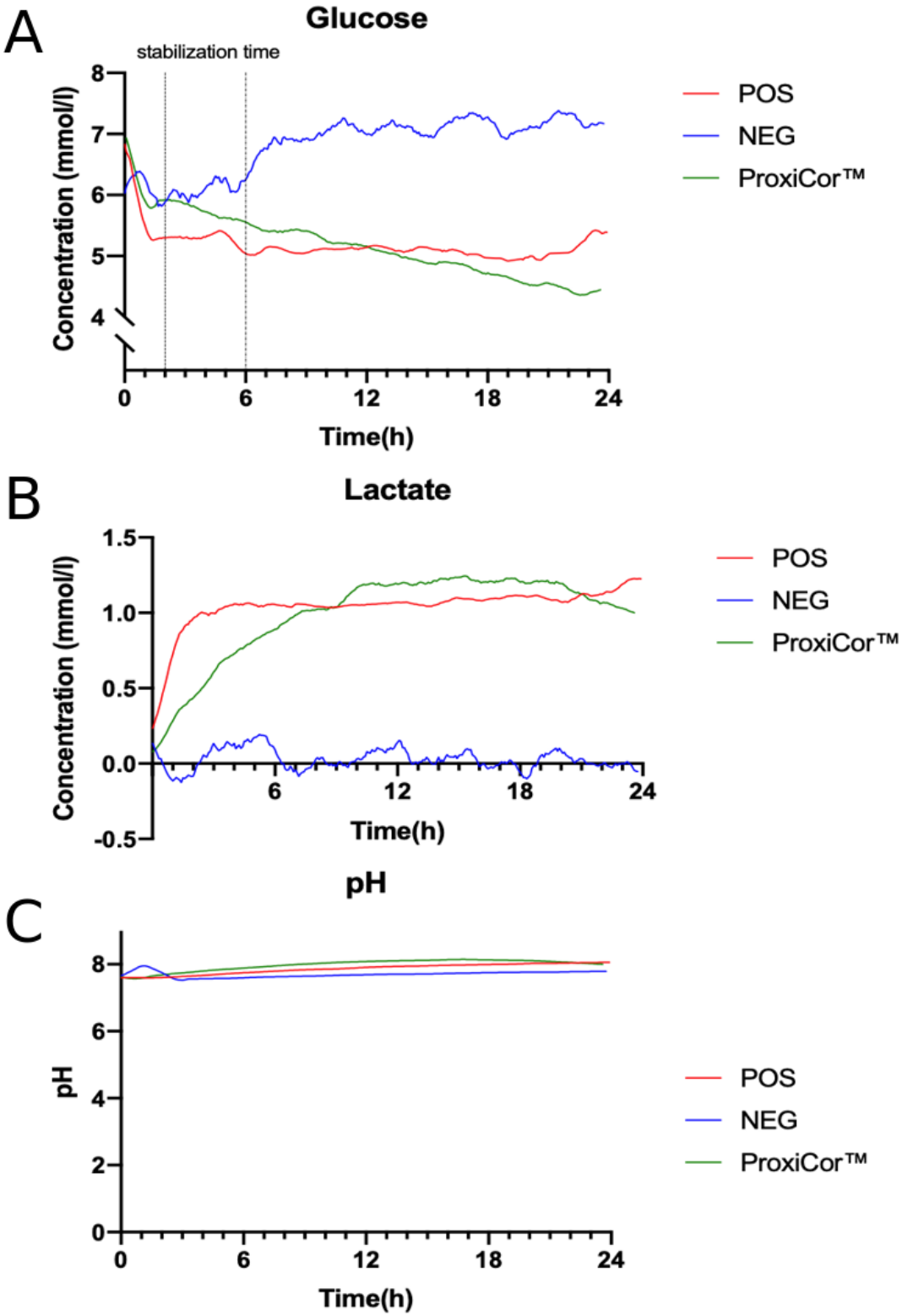

Figure 9. A. Real-time measurements of glucose concentrations (mM) and B. lactate concentrations (mM) in the receptor chamber. In the biosensor, there was three pins for measure lactate and only one pin for measure glucose. Thus, the stabilization time of the biosensor affected the glucose measurement more than the lactate measurement, which was calculated as a mean value of all three pins. The stabilization time varied from 2 to 6 hours and is presented in the glucose graph. C. pH stability in the donor chamber.

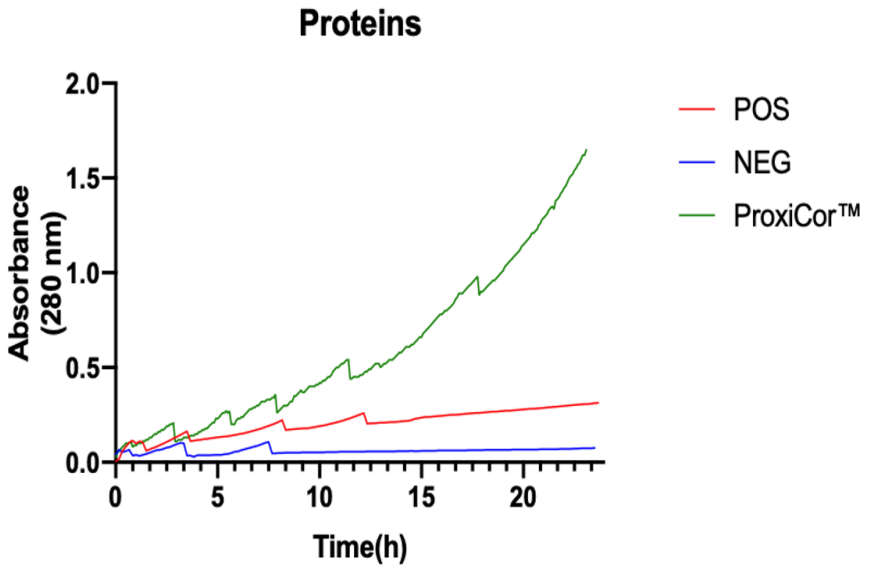

Figure 10. Changes in absorbance at 280 nm with ProxiCor™ , positive control and negative control. As the absorbance measurements with ProxiCor™ were higher than with positive control, the amount of proteins passed was not directly readable from the change in absorbance.

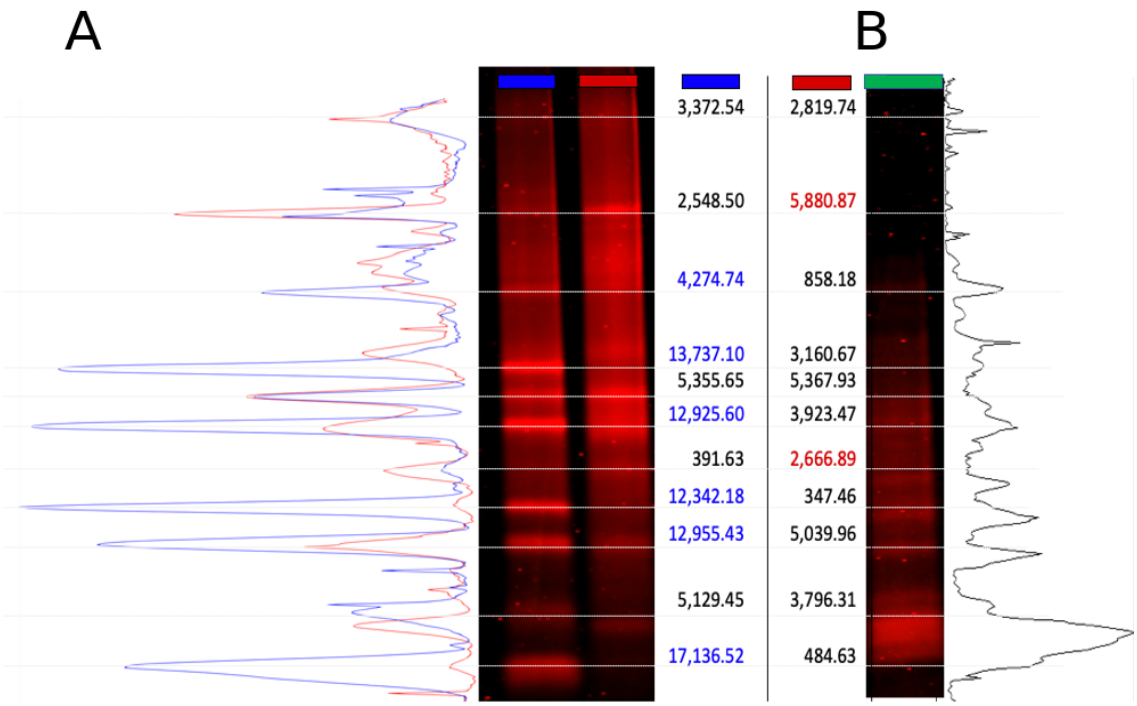

Figure 11. A. After incubation with ProxiCor™, the samples were collected from the donor chamber (blue) and receptor chamber (red) and run through SDS-page. The values present the areas of the histogram bars and values that differ between chambers are highlighted. Blue ones are overrepresented in the donor side and red ones are overrepresented in the receptor side. B. To confirm an assumption that ProxiCor™ itself releases proteins, the membrane itself was incubated in NaCl for 24 hours and concentrated sample (green) was run through SDS-page.

4.2. Effect of biomembrane permeability on cell viability and proliferation

To study the effects of biomembrane permeability on cell viability and proliferation, the optimal conditions were determined. The optimal conditions were considered as conditions where the serum addition had the greatest effect on cell cycle phases after starvation period. Although the viability decreased as serum starvation prolonged, the effects of serum additions on cell cycle phases enhanced. The greatest effects can be detected after 7 days serum starvation and 10 % serum addition. The viability measurements and cell cycle phase analyses of different test conditions are presented in Figure 12.

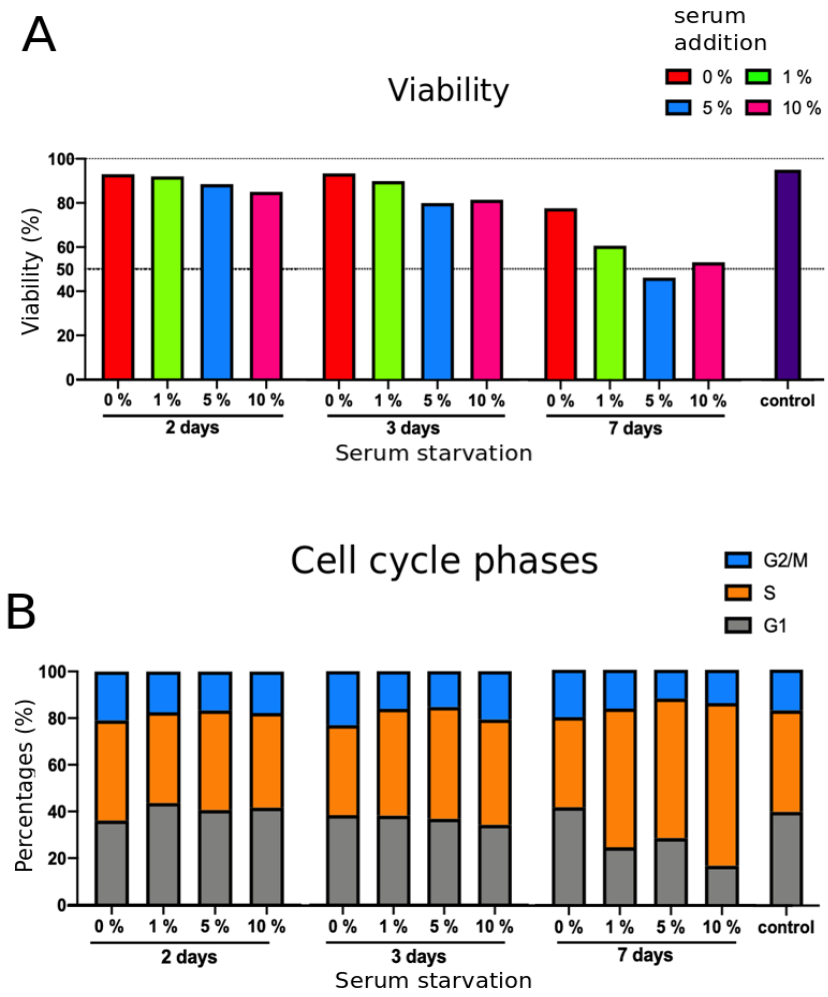

Figure 12. A. Cell viability measurements after varying serum starvation days and FBS additions of varying strengths. B. Cell cycle phase measurements after different test conditions. A normal cell culture without serum starvation and 5 % FBS addition was considered as a control for A and B.

The effects of biomembrane permeability on cell viability and proliferation were examined for ProxiCor™ and negative control membranes (Figure 13). As the ProxiCor™ was found to be permeable, no positive control membrane was investigated. Based on the examination of the optimal conditions, the HEK-293 cell solution in Leibovitz L-15 media was inserted to the donor chamber after 7 days serum starvation and the Leibovitz L-15 media with 10% FBS addition was inserted to the receptor chamber. The samples were collected and analyzed after 24 hours incubation. pH in the donor chamber and the receptor chamber was also measured after incubation to study the stability of pH. Use of ProxiCor™ allowed serum access to the cells, which was reflected in reduced viability. However, the pH was leveled with ProxiCor™ as it enables the movement of small molecules. With impermeable membrane, most of the cells remained in G1 phase, while with ProxiCor™ the serum accessibility enhanced the cell proliferation and increased the number of cells in S phase.

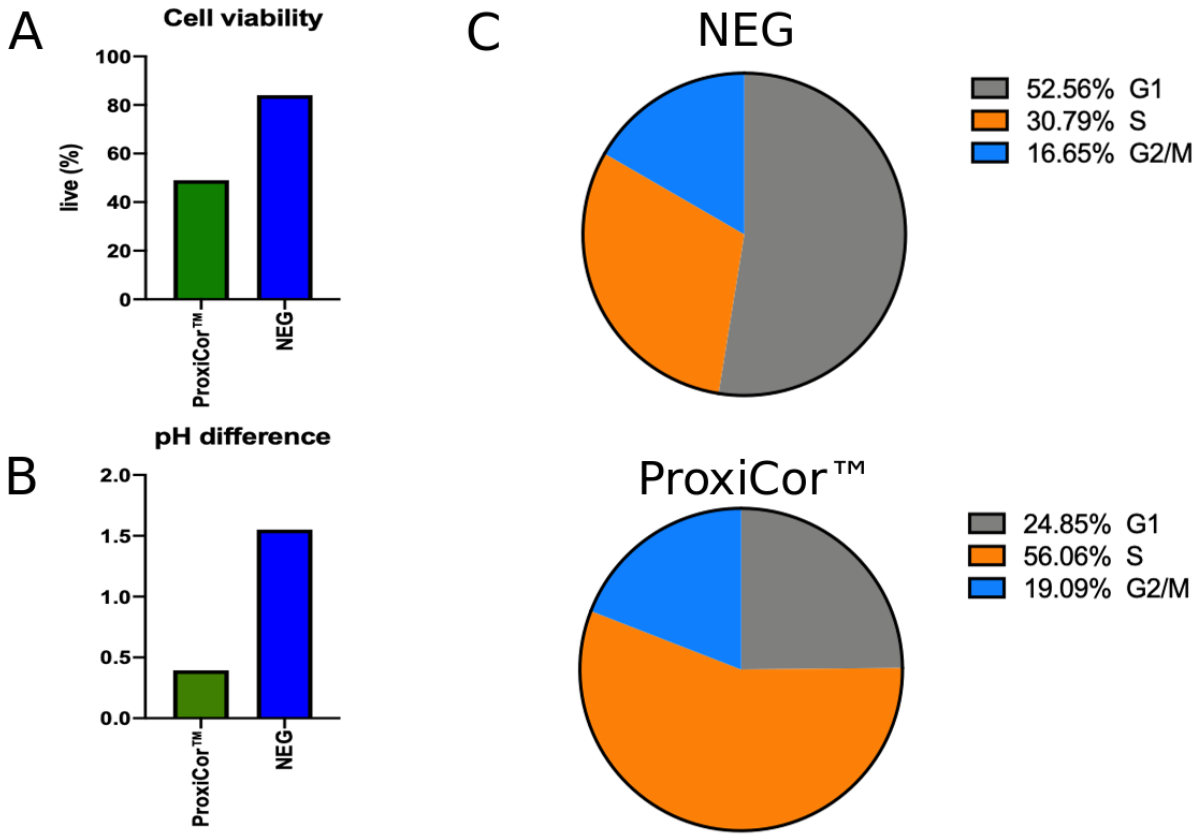

Figure 13. A. Effects of 24 hours incubation with 10% FBS to cell viability with permeable ProxiCor™ and impermeable membrane (NEG). The bars present the percentage of trypan blue negative cells, which were considered as live cells, in cell suspension. B. The difference in pH between the donor and the receptor chamber with ProxiCor™ and impermeable membrane. C. Distribution of the cell cycle phases after the incubation.

The ProxiCor™ membrane was studied also without the FBS addition to investigate whether the proteins, released from the membrane itself, affect the cell cycle. To exclude the effect of small molecule movement, a dialysis membrane with MWCO value of 0,1-0,5 kDa was examined alongside. Although the number of cells in S phase slightly increased compared to impermeable membrane, the results did not differ between the ProxiCor™ and the dialysis membrane. Thus, the proteins released from ProxiCor™ do not in themselves promote cell proliferation. Results are presented in Figure 14.

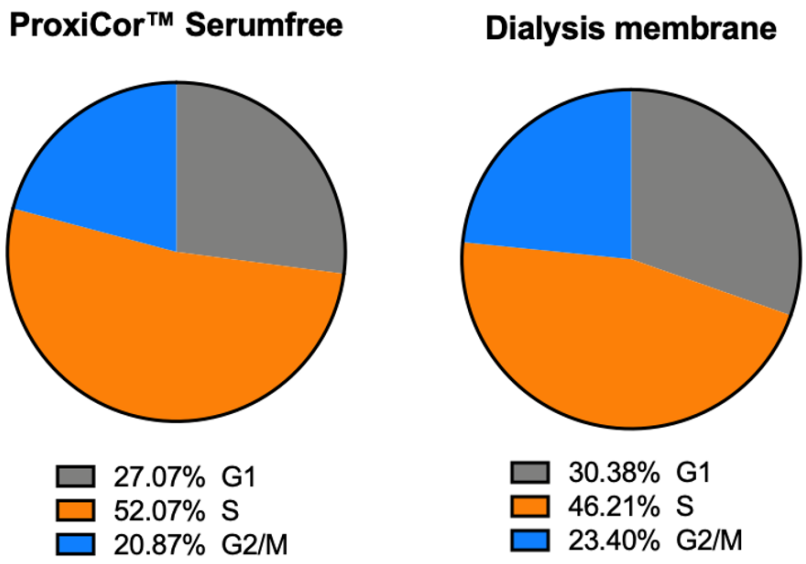

Figure 14. The ProxiCor™ was also investigated without the serum addition to determine the effects of protein released from the membrane itself. The effects of dialysis membrane with 0,1-0,5 kDa molecular weight cut-off (MWCO) was investigated alongside.

5. Discussion

5.1. The properties of ProxiCor™

The built test set up was evaluated by investigating the properties of the ProxiCor™ membrane. Unlike expected, the ProxiCor™ membrane resulted to be permeable both for proteins and small molecules. The movement rates of small molecules were slower with ProxiCor™ membrane compared to positive control of 0.4 µm pores, but the movement was still effective. With small molecules such as lactate, which has a molecular weight (MW) of 90 Da, the equilibrium could be detected within 10 hours and with larger molecules, such as glucose (MW 180 Da), within 13 hours.

The protein movement through ProxiCor™ was not reliably measurable with the spectrophotometer, since the membrane itself degraded and released proteins. However, the SDS-page revealed that ProxiCor™ is also permeable to proteins. Protein size did not affect the permeability within the used range, but some affecting factors were present since the distribution of proteins was not similar in both sides of the membrane. This might be due the 3D architecture or the charge of the protein. The identification of the unequally distributed proteins might provide us with important information about the factors affecting the protein permeability through the ProxiCor™ biomembrane. This information could be utilized in the development of an improved membrane with selective permeability with which the secreted proteins could be retained under the membrane and the beneficial effects of the transplanted cells' or micrografts' secretome could be maximized.

In 2004, Sariakaya et al. and Li et al. reported beneficial effects of SIS-ECM degradation products to host responses. However, the cell cycle analyses between a ProxiCor™ and a dialysis membrane, which allows only small molecule movement, did not differ, while the movement of small molecules slightly induced the cell proliferation compared to impermeable membrane. The lactate measurements after incubations (data not shown) also indicated that acceleration in cell metabolism, which can be detected by increase in lactate production, is dependent on the presence of the serum and not the ProxiCor™ itself or the dialysis membrane elicit the response.

Even if the tissue is not serum-starved during AAMs patch preparation, the processing does expose the tissue and micrografts to stressful conditions. The stress upon transplantation and exposure to tissue and pericardial fluid can be compared, at least to some extent, to stress from serum restimulation, which has been reported in several studies to cause cell death. In 2015, Gao et al. reported active ferrotopsis when adding serum to the cell culture after serum and amino acid starvation. However, in their experiments the restimulation with dialyzed FBS containing only small molecules of serum did not enhance cell death. In addition, the reports from Sarikaya et al. (2004) and Li et al. (2004) highlighted the importance of low molecular weight proteome. In the report of Sariakya et al., the antibacterial effects from SIS-ECM were due the low-molecular weight fractions from 5 to 16 kDa, while the molecular weight of released components from SIS-ECM varied from less than 4 kDa to over 100 kDa. Li et al. also reported same molecular weight fraction to activate the recruitment of heart endothelial cells *in vitro*. Overall, the low-molecular weight serum proteome is attracting increasing interest in proteomics. The most abundant proteins in serum range from 50 kDa to 100 kDa comprising over 99% of the whole serum proteome (Bellei et al., 2011). Figure 15 presents several molecules which are known to participate in proliferation,

angiogenesis and cell migration as well as inflammatory responses. The cut-off of high-molecular weight proteome (MW over 50 kDa) may inhibit or decrease the shock of processed cells while retaining the positive outcomes of AAMs patch. This is something that should be addressed in further investigations.

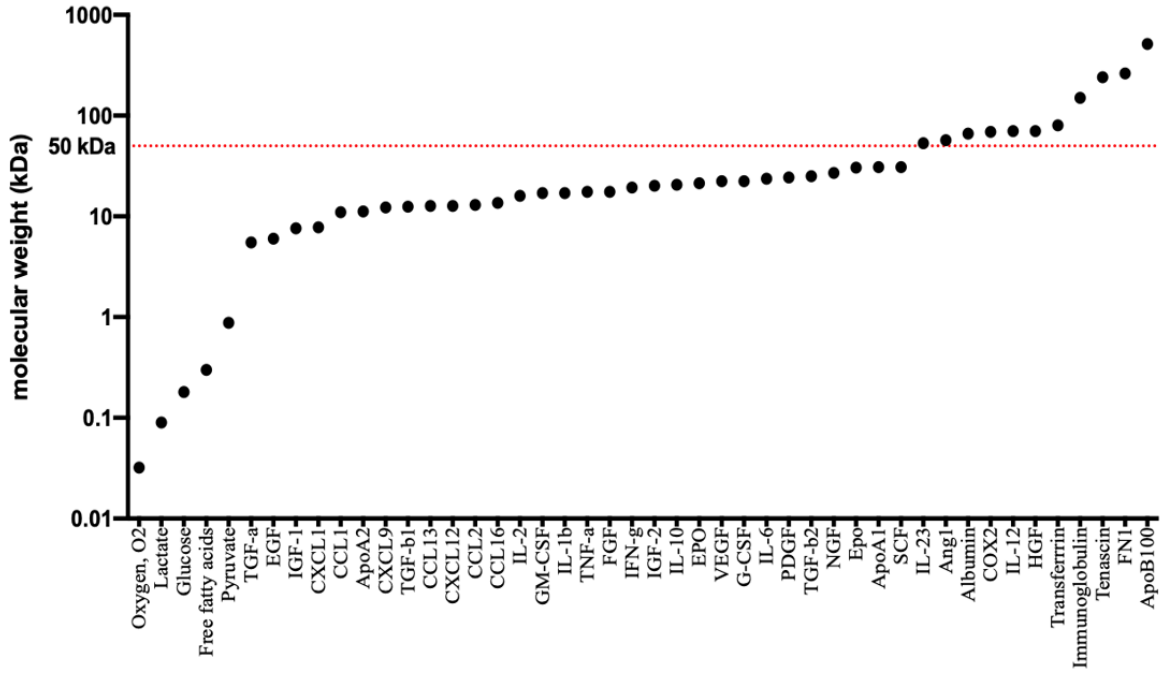

Figure 15. Molecular weights (MW) of molecules affecting the cell proliferation and viability. The drop off MW over 50 kDa (marked as a red line) may inhibit or decrease the shock and thus be beneficial to cellular responses .

5.2. Advantages of the test set up

The aim of this Master's thesis was to build a test set up to study biomembrane properties. The results on ProxiCor™, proved the functionality of the test set up. While studying the optimum properties for epicardial patch, the test set up can be utilized for several research approaches in the fields of biomembrane development, *in vitro* studies or measurement methodology development (Figure 16).

The test set up can support tissue engineering, which determines the biomembrane design and properties. Biomembranes should share the native three-dimensional (3D) structure, surface properties and mechanical strength of heart tissue. As a material, synthetic or natural polymers set

different challenges to tissue engineering. Synthetic polymers can be fabricated homogeneously, and the scaffold structure can be designed to achieve improved mechanical tolerance with desired porosity and surface properties. However, this can lead to reduced bioactivity. Scaffolds of biological origin, usually composed of ECM or purified individual components of it, are usually considered to be biodegradable and biocompatible. The challenges with natural polymers are that homogenous and reproducible structures can be challenging to achieve. (Pattar, Hassanabad & Fedak, 2019.) Traditionally fibers have been cultured with cells to form a scaffold (Carrier et al., 1999), but more advanced ways to control the scaffold manufacturing are electrospinning and rotary jet spinning. In electrospinning, micro- or nano-sized fibers are created by charging the polymer stream electrically and these fibers are then collected to form a scaffold. Electrospun membranes, made for example from PLA, polyurethane or albumin fibers, have been shown to be promising scaffold materials for cardiac-tissue engineering (Hosseinkhani et al., 2010; Kaiser et al., 2017; Fleischer et al., 2017). While hunting for the perfect biomembrane for the epicardial patch, the effects of scaffold design to diffusion properties and cell responses are easily compared with our standardized test set up.

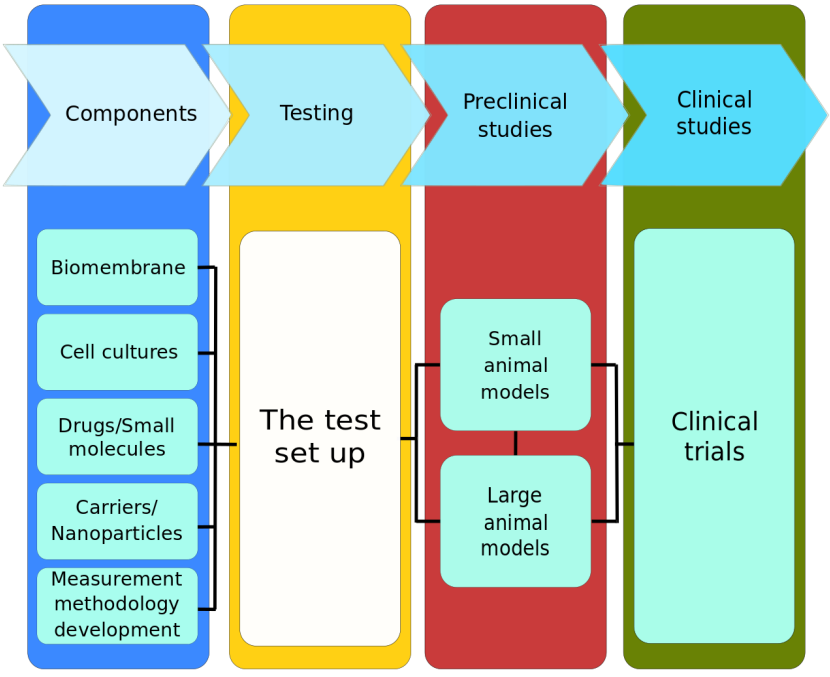

Figure 16. The biomembrane test set up, which was built in this Master's thesis, enables several research possibilities before entering the preclinical or clinical phases. Not only biomembrane permeability can be tested, but also the degradation of biomembranes and cell responses to degradation products can be examined. With appropriate arrangements, cell responses to biomembranes with loaded nanoparticles or small molecules can be studied real-time. There also is a constant need for new real-time measurement methods, which can easily be tested with described system.

Besides to permeability testing, the test set up offers a possibility to study the degradation of a given biomembrane and examine how the degradation products affect cells. In addition to the degradation products from biomembrane itself, a rising trend is to load biomembranes with small molecules, drugs or nanoparticles, which release during degradation. The effects of released molecules can be studied real-time or at different time points and the experiments are easily repeated with different solutions or cell types. The gained information on how different cell types respond to different stimuli, can be used to develop the biomembrane or patch treatments towards a more personal direction. Not all patients can be studied individually, but the cell responses to treatment can be studied among different patients' groups with different disease backgrounds. The effects of the environment can be easily examined by moving the test set up into the desired environments, for example hypoxic conditions can be achieved by moving the system into an oxygen-level-controlled incubator.

Currently, further studies on the proteome secreted by engrafted cells, is not feasible due to the requirements of needed protein amounts and issues related to overloaded proteins, such as albumin or immunoglobulins, in the sample. The test set up offers a possibility to concentrate the proteins by changing the media in the receptor chamber without disturbing the cells in the donor chamber. By providing energy sources and removal of end-products of cellular metabolism, the test set up enhances the cell wellbeing and provides favorable conditions for increased secretion of proteins. While retaining the secretome in the donor chamber, the concentration of secreted proteins increases and can thus allow further investigations.

5.3. Limitations and improvements of the test set up

This first version of the test set up serves as a solid background for further modifications to yield results from different biomembranes and cell types as well as to integrate additional measurements to further characterize both the biomembrane properties as well as the cellular metabolic responses.

The flow cell-based biosensor, with individual pump, is susceptible for disturbance. This can be detected as sudden increases and decreases in glucose and lactate concentrations (Figure 9). Instead of using flow cell-type biosensors, there are strip-based sensors, which can be inserted straight to the chambers, and the use of individual pumps can be avoided. This type of direct measurement could stabilize the biosensor faster in the beginning of the test and also stabilize the real-time

measurements. However, as with all biosensors, it must be ensured that no harmful agents are released from the sensor to affect the cells in the test set up. The absence of the pump also affects the liquid amount in a system, since in the tubing there are always a dead volume, which affects the concentration gradient between chambers. The used buffer solution, PBS, was not recommended by the biosensor manufacturer, instead it could be replaced with a bicarbonate buffer to achieve more stable measurements.

The temperature has a great effect on diffusion rates and biological responses, and it should also be measured in real-time. The currently used pH meter has a temperature sensor, but the fluid level in the chamber is too low to get accurate results. One possibility is to replace the currently used pH meter with another sensor, which could measure the temperature from small liquid amounts. In addition, some biosensors have the possibility to measure temperature and that might be another way to add the real-time temperature measurement to the test set up.

Currently, the tubing, from the receptor chamber to the spectrophotometer and back, is long and wide and thus the dead volume in the system increases the liquid volume in the receptor chamber to approximately 1 ml. As with the biosensor, this affects the concentration gradients of the system. In the future, a smaller spectrophotometer with shorter, thinner tubing could provide more accurate results. The smaller size of the spectrophotometer could also improve mobility of the test set up. For more precise follow up of protein movement, there could be more than just one spectrophotometer connected. A sample, taken at certain time points, could be led to a branch that divides the sample into several portions. Each portion could then be passed through a membrane of varying MWCO and then be detected with a single wavelength (280 nm) spectrophotometer. This could give us more information about the size distribution of proteins. Size exclusion liquid chromatography would probably give more accurate results with smaller sample size, but it is difficult to place it in the current system without giving up the advantages of the system, such as easy mobility.

HEK-293 cells were used in this study because they are easy to grow and are commonly used in research as model cells. These cells differ in many ways from cells of the cardiac tissue. For example, the levels of the cell surface transporter proteins for glucose, lactate and fatty acids, as listed in Table 2, are very different in the HEK-293 cells than in the heart tissue. This is something that should be considered while interpreting the cell responses. In addition to HEK-293 cells, cardiomyocytes grown on microcarrier beads can be inserted as a suspension to the test set up to improve comparability. Carefully regulated metabolism of cells can be achieved by optogenetically treated cardiomyocytes which also enables response studies during contraction (Pastrana, 2011;

Björk et al., 2017). Eventually, the tissue pieces from atrial appendage could be used to study heart tissue instead of just one individual cell line.

Table 2. Comparison of solute carrier transport protein gene expression levels in HEK-293 cells and in heart tissue. The proteins which are over-presented in HEK-293 cells, compared to heart tissue, are marked as red (HEK-293/Heart tissue ratio >1.5) and proteins and proteins which are under-presented in HEK-293 cells, compared to the heart tissue, are marked as blue (HEK-293/Heart tissue ratio <0.5).

	Gene name	Alternate name	HEK-293	Heart tissue	HEK-293/Heart tissue ratio
Glucose transporters	SLC2A1	GLUT1	2.60	5.00	0.52
	SLC2A2	GLUT2	0.00	0.10	0.01
	SLC2A3	GLUT3	0.20	22.80	0.01
	SLC2A4	GLUT4	1.50	37.20	0.04
	SLC2A5	GLUT5	0.00	1.40	0.00
	SLC2A6	GLUT6	0.80	3.00	0.27
	SLC2A7	GLUT7	0.00	0.20	0.01
	SLC2A8	GLUT8	6.10	9.80	0.62
	SLC2A9	GLUT9	0.10	2.70	0.04
	SLC2A10	GLUT10	0.10	6.70	0.02
	SLC2A11	GLUT11	2.90	8.50	0.34
	SLC2A12	GLUT12	6.50	11.40	0.57
	SLC2A13	GLUT13	5.40	4.90	1.10
	SLC2A14	GLUT14	6.20	0.10	61.40
Monocarboxylate transporters (lactate transporters)	SLC16A1	MCT1	20.40	39.00	0.52
	SLC16A2	MCT7,MCT8	1.30	10.70	0.12
	SLC16A3	MCT3	0.20	7.40	0.03
	SLC16A4	MCT4	1.50	4.00	0.40
	SLC16A5	MCT5	0.00	8.60	0.00
	SLC16A6	MCT6	0.20	1.40	0.14
	SLC16A7	MCT2	0.30	34.70	0.01
	SLC16A8	MCT3[2]	1.80	2.90	0.62
	SLC16A9	MCT9	1.40	1.40	1.00
	SLC16A10	MCT10	12.40	2.80	4.43
	SLC16A11	MCT11	1.00	2.40	0.42
	SLC16A12	MCT12	0.00	4.00	0.00
	SLC16A13	MCT13	2.20	1.30	1.69
	SLC16A14	MCT14	6.20	3.00	2.07
Fatty acid transporters	CD36	CD36	0.10	20.60	0.01
	SLC27A1	FATP1	0.80	18.30	0.04
	SLC27A2	FATP2	10.70	0.20	53.24
	SLC27A3	FATP3	2.30	7.00	0.33
	SLC27A4	FATP4	1.50	7.70	0.20
	SLC27A5	FATP5	14.70	1.30	11.30
	SLC27A6	FATP6	0.10	20.60	0.01

After the experimental incubation, the biomembrane itself is an important subject of studies. Currently, the imaging of nanosized pores and structures is challenging. The field emission scanning electron microscopy (FE-SEM) is usually used in the imaging of thin membranes, but the resolution (0.4 nm) is not necessarily enough. Transmission electron microscopy (TEM) has better resolution (0.2 nm), but with thin membranes the sample preparation is difficult. Atomic force microscopy (AFM) is used to study biological membranes and could be utilized for this purpose. In addition, during incubation the cells can attach to the biomembrane and thus the histological

staining of biomembrane can offer an important information about the interactions between cells and the biomembrane.

6. Conclusions

This Master's thesis describes a test set up, which can be used to experimentally test selected properties of biomembranes. The movement of small molecules and proteins are easily followed real-time together with pH recording. The test set up was evaluated by examining the properties of ProxiCor™, a biomembrane currently used in the AAMs patch. As a result, the ProxiCor™ demonstrated permeability both for small molecules and for proteins. It also stabilized pH compared to an impermeable membrane. Overall, the results prove the functionality of the test set up as an experimental tool for biomembrane characterization. The test set up can easily be further developed in compactness, mobility, cell culture incubator compatibility and incorporation of measurements of additional parameters. With the help of this set up, therapeutic biomembrane components of epicardial patches can be investigated, evaluated and their optimal requirements identified.

Cell therapies have been investigated in the treatment of heart failure since the early 1990's. Many positive outcomes have been reported but overall the results are mixed. One of the most influencing factors is the delivery of cells. Traditionally used injection-based techniques are often reported to have poor cell retention and survival. Patch-based techniques have not just shown to improve the cell retention, but also support the heart mechanically while utilizing the paracrine effects more effectively compared to injections. In addition to being just a carrier and cover for grafted cells, the biomembrane in the patch has a great impact on the cell response. In future, it is a challenge to develop a biomembrane which provides ideal environment for the grafted cells and further supports the scar tissue reverse remodeling. With described test set up we are one step closer to the perfect biomembrane and thus closer to solution of how to repair a scarred heart.

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